Attachment, internalization, and dissemination of human norovirus and animal caliciviruses in hydroponically grown Romaine lettuce

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Abstract

Fresh produce is a major vehicle for the transmission of human norovirus (NoV) because it is easily contaminated during both pre- and post-harvest stages however, the ecology of human NoV in fresh produce is poorly understood. In this study, we determined whether human NoV and its surrogates can be internalized via roots and disseminated to edible portions of the plant. The roots of Romaine lettuce growing in hydroponic feed water were inoculated with 1×10^6RNA copies/ml of human NoV GII.4 strain or $1\text{-}2\times10^6$PFU/mL of animal caliciviruses (Tulane virus, TV; and murine norovirus, MNV-1), and plants were allowed to grow for 2 weeks. Leaves, shoots, and roots were homogenized and viral titers and/or RNA were determined by plaque assay and/or real-time RT-PCR. For human NoV, high levels of viral genome RNA (10^5-10^6 RNA copies/g) were detected in leaves, shoots, and roots at day 1 post-inoculation and remained stable over the 14 day study period. For MNV-1 and TV, relatively low levels of infectious virus particles (10^1-10^3 PFU/ml) were detected in leaves and shoots at days 1 and 2 post-inoculation, but reached a peak titer (10^5-10^6 PFU/g) at days 3 or 7 post-inoculation. In addition, human NoV

had a rate of internalization comparable with TV as determined by real-time RT-PCR. Whereas, TV was more efficiently internalized than MNV-1 as determined by plaque assay. Taken together, these results demonstrated that human NoV and animal caliciviruses attached tightly to roots, became internalized via roots, and efficiently disseminated to the shoots and leaves of the lettuce.

Introduction

The Caliciviridae family includes a number of enteric viruses that cause gastroenteritis in humans and animals. Examples of these viruses include human norovirus (NoV), human sapovirus, and the newly discovered monkey calicivirus (Tulane virus, TV). Human NoV is the leading cause of nonbacterial gastroenteritis worldwide, contributing to over 95% of all non-bacterial acute gastroenteritis each year, and more than 60% of all foodborne illnesses reported annually (Atmar et al.,2008). The virus is highly infectious, resistant to common disinfectants, and has a low infectious dose (Wei et al., 2010, 2011). However, human NoV remains difficult to study because it cannot be grown in cell culture and it lacks a small animal model (Atmar et al., 2008). For these reasons, human NoV is classified as a Category B biodefense agent by the National Institute of Allergy and Infectious Diseases (NIAID).

In recent years, the consumption of fresh produce has increased as individuals strive to maintain a healthy diet. However, disease surveillance has shown that vegetables and fruits are major vehicles for the transmission of human NoV, since they normally undergo little or no processing and are easily contaminated pre- and post-harvest through irrigation, fertilizers, soil, wildlife, domestic animals, packaging, and food handlers (Abbaszadegan et al., 2003, Doyle and Erickson, 2008, Heaton and Jones, 2008, Lynch et al. 2009, Rawsthrone et al. 2009). It has been

reported that norovirus accounts for more than 40% of outbreaks caused by fresh produce in the US annually (Seymour, 2001). Fresh-produce related outbreaks caused by noroviruses have been reported in lettuce, salad, fruit salad, tomato, carrot, melon, strawberry, raspberry, orange juice, fresh cut fruit, coleslaw, spring onion, and other vegetables (Abbazadegan, 2003, Doyle and Erickson, 2008, Heaton and Jones, 2008, Lynch et al., 2009, Rawsthorne et al., 2009). In another survey it was found that, salads, lettuce, and fruits contributed 67%, 47%, and 67% respectively, to human norovirus gastroenteritis in the US from 1990-2005 (DeWaal and Bhuiya, 2007). Increasing outbreaks of viruses in fresh produce gives high urgency to understanding the ecology of enteric viruses in vegetables and fruits and the mechanism of viral contamination and persistence in fresh produce.

Internalization of pathogens is considered one of the major routes for contamination of fresh produce. It has been well established that foodborne bacterial pathogens such as *E. coli.* O157:H7 and *Salmonella* sp., become internalized and disseminated in plant crops, including lettuce, spinach, tomato, and mung bean shoots via the plant root systems, through wounds in the cuticula, or through stomata (Bernstein et al., 2007, Jablasone et al., 2005, Aruscavage et al., 2008). The efficiency of the internalization of bacterial pathogens in plants can be affected by many factors such as the type of plant, plant stress, bacterial species and strains, bacterial dose, and environmental humidity and temperature (Bernstein et al., 2007, Doyle and Erickson, 2008, Jablasone et al., 2005, Seymore and Appleton, 2001, Aruscavage et al., 2008). However, the penetration, uptake, internalization, dissemination, and persistence of foodborne viruses in plants is poorly understood. The feasibility of internalization of human enteric viruses by plants is supported by the ability of plants to internalize their own viral pathogens, which can be taken up from soil and water. As the size of a virus is approximately 1000 times smaller than bacteria, in

theory, the efficiency of a smaller pathogen to enter and disseminate in plants would be elevated. Since human enteric viruses may be present in sewage-contaminated soil or water, they may also be taken into the plant through the roots and/or leaves. The dissemination of the viruses via the vascular system of the plant could also facilitate movement of the virus from the inedible portions of the plant (roots) to the edible portions of the plant (leaves).

To date, only two studies have examined whether human NoV and its surrogates can be internalized and disseminated in plants. Urbanucci et al., (2009) found that canine calicivirus (CaCV) RNA could be detected in the aerial tissues of lettuce grown both hydroponically and in soil, though not all samples in the treatment groups tested positive. In contrast, when a human NoV G.II strain was used under the same experimental conditions; no viral RNA in the lettuce was detected even when challenged with a high level of human NoV (Urbanucci et al.,2009). Most recently, Wei et al., (2011) found that less than 2 logs of infectious MNV-1 could be detected in leaf samples from days 1 to 5 when the roots were challenged with high level of MNV-1 (5×10^{8}PFU/ml). However, no infectious virus was detected when the roots were challenged with low level of MNV-1 (5×10^{5}PFU/ml). Furthermore, infectious MNV-1 was undetectable when lettuce was grown in soil even inoculated with high level of MNV-1 (5×10^{8}PFU/ml) (Wei et al., 2011). These two studies demonstrated that low levels of virus internalization of human NoV surrogates, such as MNV-1 and CaCV, can occur in growing lettuce. However, based on Urbanucci's 2009 study, it seems that human NoV cannot be internalized via roots and disseminated to leaves of lettuce. The basis for the differences seen in the rate of internalization between human NoV and its surrogates has not been elucidated.

The objectives of this study were to determine the attachment of human NoV to the roots of lettuce and to evaluate the internalization and dissemination of human NoV in hydroponically

growing lettuce using a GII.4 human NoV strain, which is currently the prevalent strain circulating in many countries. In addition, we compared the efficiency of viral internalization and dissemination of different caliciviruses (MNV-1, TV, and human NoV) in lettuce.

Materials and Methods

Viruses and cell culture

Murine norovirus strain MNV-1 was generously provided by Dr. Herbert W. Virgin IV, Washington University School of Medicine. Tulane virus was a generous gift from Dr. Xi Jiang at Cincinnati Children's Hospital Medical Center. MNV-1 and TV were propagated in confluent monolayers of the murine macrophage cell line RAW 264.7 and the monkey kidney cell line MK2-LLC (ATCC, Manassas, VA), respectively. RAW 264.7 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invirogen), at 37^{o}C in a 5% CO_2 atmosphere. For growing MNV-1 stock, confluent RAW 264.7 cells were infected with MNV-1 at a multiplicity of infection (MOI) of 0.1. After 1 h incubation at 37°C, 15 ml of DMEM with 2% FBS was added. The virus was harvested 2 days post inoculation by three freeze-thaw cycles and low speed centrifugation at 1000× g for 30 min. MK2-LLC cells were cultured in low serum Eagle's minimum essential medium (Opti-MEM), supplemented with 2% FBS, at 37^{o}C in a 5% CO_2 atmosphere. For growing TV stock, MK2-LLC cells were washed with Hank's balanced salt solution (HBSS) and subsequently infected with TV at an MOI of 0.1. After 1 h incubation at 37 °C, 15 ml of Opti-MEM with 2% FBS was added. The virus was harvested 2 days post

inoculation and subjected to three freeze-thaw cycles, followed by centrifugation at 1006 × g for 30 min.

Plant cultivation for hydroponic growth

Seeds of romaine lettuce (Lactuca sativa) were planted in 2 inch plug trays and grown under greenhouse conditions. Twenty days after germination, plants were removed from the soil and inserted in the hydroponic growth system. The hydroponic feed water was supplemented with a nutrient solution containing nitrogen, phosphorus, and potassium. The feed water was also supplemented with 1% penicillin, kanamycin, and streptomycin to control microbial growth. After viral inoculation, the plants were grown in the lab under a fluorescent light cycle of 12 hours light and 12 hours darkness. The temperature and relative humidity was maintained at 20^{o}C and 40%, respectively.

Viral inoculation and sample collection

The hydroponic feed water was inoculated with either MNV-1 or TV. The total volume of the hydroponic feed water reservoir was 100 ml which was inoculated with 5 ml of viruses having a starting titer of 1×10^{6} PFU/ml. Controls received no viral inoculation in feed water. At days 0 (before viral inoculation), 1, 2, 3, 7, and 14 the leaves, shoots, and roots were harvested and weighed. The samples were homogenized by freezing with liquid nitrogen and grinding with a mortar and pestle. Homogenized tissue was resuspended in 5 ml phosphate buffered saline (PBS, pH 7.0). Sample homogenates were centrifuged at 1000 × g to remove cellular debris and the virus containing supernatant was transferred to a new collection tube for viral enumeration by plaque assay. At days 0, 1, 2, 3, 7, and 14, 500 µl samples of feed water were collected for

determination of viral titer by plaque assay. For chlorine treated samples, following harvest each tissue was submerged in a 50 ml conical tube containing 1000 ppm chlorine and incubated at room temperature for 5 min. After chlorine wash, samples were placed in a new 50mL tube containing tap water and submerged for 5 min with gentile agitation. Following tap water wash, samples were placed in a 50 ml tube containing 0.25 M sodium thiosulfate to neutralize residual chlorine. All solutions were changed between samples to maintain the oxidation potential of the chlorine solution. Samples were then homogenized and processed as described above. For human NoV, the feed water was inoculated to a starting concentration of 1×10^6 RNA copies/ml, while controls received no viral RNA. Sample collection methods were the same as above. Quantification of viral genomic RNA was executed using RT-qPCR.

Virus enumeration by plaque assay

MNV-1 and TV were quantified by plaque assay in RAW 264.7 and LLC-MK2 cells, respectively. Briefly, cells were seeded into six-well plates (Corning Life Sciences, Wilkes-Barre, PA) at a density of 2×10^6 cells per well. After 24 h incubation, RAW 264.7 and MK2-LLC cell monolayers were infected with 400 µl of a 10-fold dilution series of MNV-1 or TV, respectively, and the plates were incubated for 1 h at 37^oC with gentile agitation every 10 min. The cells were overlaid with 3 ml of Eagle's minimum essential medium (MEM) containing 1% agarose, 2% FBS, 1% sodium bicarbonate, 0.1 mg kanamycin/ml, 0.05 mg gentamicin/ml, 15 mM HEPES (pH 7.7), and 2 mM L-glutamine. After incubation at 37 °C and 5% CO2 for 2 days, the plates were fixed in 10% formaldehyde. The plaques were visualized by staining with 0.05% (w/v) crystal violet. Viral titer was expressed as mean log10 plaque forming unit (PFU)/ml ± standard deviation.

Quantification of viral RNA by real-time RT-PCR

Since human NoV cannot be grown in cell culture, real-time RT-PCR was used to quantify viral genomic RNA copies. Briefly, total RNA was extracted from samples using an RNeasy Kit (Qiagen), followed by reverse transcription and real-time PCR. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer VP1-P1 (5'-TTATAATACACGTCTGCGCCC-3'), which targets the VP1 gene of human NoV. The VP1 gene was then quantified by real-time PCR using custom Taqman primers and probes (Forward primer: 5'-CACCGCCGGGAAAATCA-3') (Reverse primer: 5'-GCCTTCAGTTGGGAAATTTGG-3') (Reporter: 5'-FAM-ATTTGCAGCAGTCCC-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems, Foster City, CA). PCR reaction and cycling parameters followed the manufacturer's protocol (Invitrogen). Briefly, TaqMan Fast Universal Master Mix was used for all reactions. For cycling parameters, a holding stage at 95°C was maintained for 20 seconds prior to cycling, followed by 50 cycles of 95°C for 1 second for annealing and 60°C for 20 seconds for extension. Standard curves and StepOne Software v2.1 were used to quantify genomic RNA copies. Viral RNA was expressed as mean log10 genomic RNA copies/ml ± standard deviation.

To compare the internalization rate between human NoV and Tulane virus, Tulane virus RNA was also quantified by RT-qPCR. First strand cDNA was synthesized by SuperScriptase III (Invitrogen) using the primer TVRT (5'-AATTCCACCTTCAACCCAAGTG -3'), which targets the VP1 gene of Tulane virus. The VP1 gene was then quantified by real-time PCR using custom Taqman primers and probes (Forward primer: 5'-TTGCAGGAGGGTTTCAAGATG-3') (Reverse primer: 5'-CACGGTTTCATTGTCCCCATA-3') (Probe: 5'-FAM-

TGATGCACACATGTGGGA-NFQ-3') on a StepOne real-time PCR machine (Applied Biosystems). PCR reaction, cycling parameters, and quantification method were identical to those used with human NoV.

RNase treatment of lettuce tissues

Following harvest, processed Romaine lettuce samples were stored at -80°C. Samples were then thawed and 100µl aliquots were incubated with (0.5µg/µl) of RNase (Invitrogen) at 37°C for 1 hr. Samples were subjected to RNA extraction using the RNeasy Kit (Qiagen), followed by real time RT-PCR using the procedures listed above.

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed by one-way multiple comparisons using Minitab 16 statistical analysis software (Minitab Inc., State College, PA). A P value of <0.05 was considered statistically significant

Results

TV was efficiently internalized and disseminated in Romaine lettuce grown hydroponically

The TV feed water in the reservoir for Romaine lettuce hydroponic growth had a starting titer of 1.25×10^6 PFU/ml. To prevent contamination, the leaves, shoots, and roots of lettuce were harvested separately at days 0, 1, 2, 3, 7, and 14 after viral inoculation (Fig.1). The kinetics of the internalization and dissemination of TV was monitored. TV was detected in leaves as soon as day 1 post-inoculation with an average titer of 6.1×10^1 PFU/g. The viral titer in the

leaves gradually increased through day 14 (Fig. 2). At day 7 post-inoculation, the viral titer reached 9.8×10^5 PFU/g, which was significantly higher than days 1, 2, and 3 (Fig. 2). The TV titer in the leaves on day 14 was 6.3×10^5, which was comparable to day 7 (Fig. 2). Similarly, infectious TV was also detected in the shoots on all days tested, with a viral titer in the shoots of 7.8×10^3 PFU/g on day 1 (Fig. 2). The viral titer gradually increased and reached a peak titer of 2.4×10^6 PFU/g on day 7. The TV titer in the shoots on day 14 was 1.3×10^6, which was a slight decrease compared to day 7. During the experimental period, the viral titer in shoots was significantly higher than that in leaves ($P<0.05$). As expected, TV was detected lettuce roots since they were in direct contact with virus-contaminated feed water. On day 1, the titer in roots was 1.5×10^5 PFU/g, and increased in titer until day 14 (Fig. 2). The TV titer in the roots on day 7 and 14 was 1.2×10^6 PFU/g and 1.0×10^6 PFU/g, respectively., and the viral titer in shoots at day 7 was higher than that found in roots ($P<0.05$). These results suggest that TV efficiently attached to roots, internalized in roots, and disseminated into shoots and leaves of the lettuce. Concurrently, the titer of the feed water was also monitored each day until the plants were harvested. Consistent with the increasing viral titer in lettuce, the titer of the TV in the feed water gradually decreased during the experimental period. On days 1, 2, 3, 7, and 14, the titer of the feed water was 3.75×10^5, 7.5×10^5, 3.5×10^4, 7.5×10^4, and 5.0×10^3 PFU/ml, respectively. To further confirm that the decreasing titer in feed water was due to internalization via roots and not to the instability of TV in feed water, TV was diluted in feed water (without lettuce) and viral titer was monitored until day 14. TV was found to be highly stable in the feed water alone over the 14 day period with no significant reduction in viral titer (data not shown). Taken together, these results suggest that TV was internalized via roots and disseminated to shoot and leaf portion of the plants.

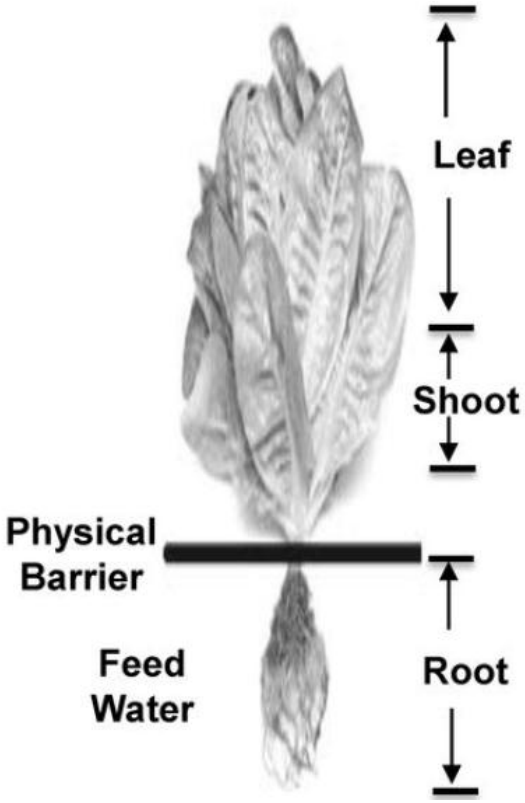

Figure 1. Schematic of harvesting procedure of Romaine lettuce. Leaf tissue represents the aerial tissues of the lettuce starting 2 inches above the root juncture and was harvested first. Shoot tissue represents the 2 inch portion of the aerial tissue connected to the root juncture and was not in contact with the feed water, which was harvested second. Root tissue consists of all lettuce roots and was in direct contact with the feed water and was harvested third.

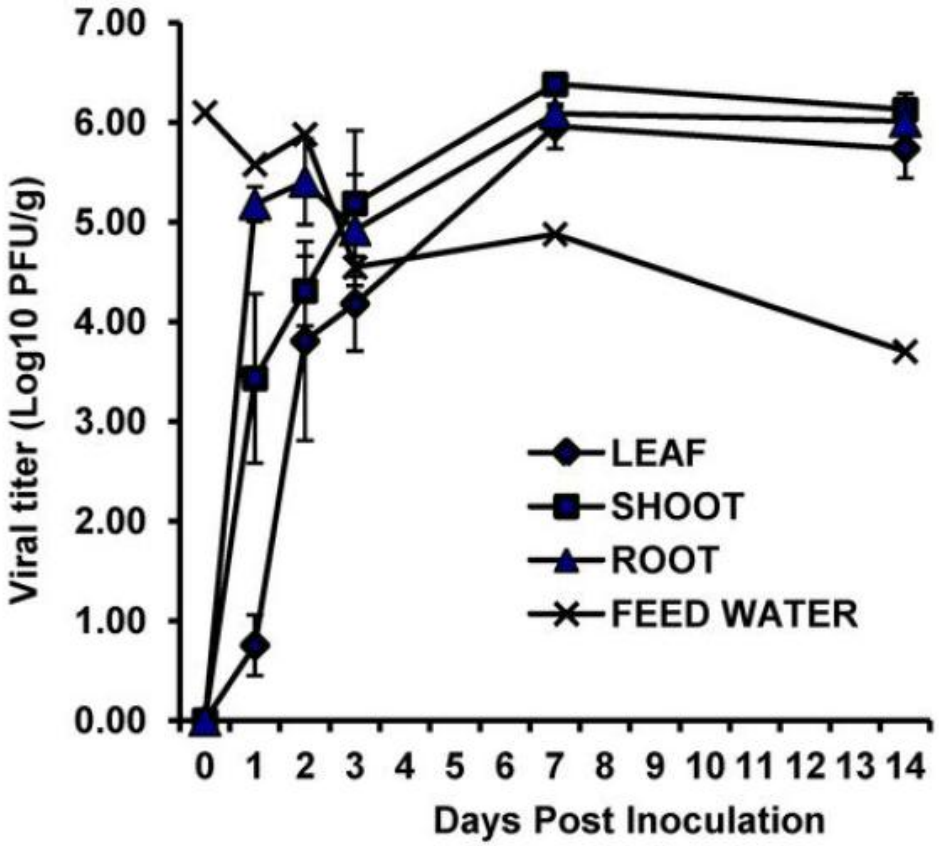

Figure 2. Internalization of TV in Romaine lettuce grown hydroponically. Viral titer is reported as PFU/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

As the plants were grown hydroponically, it is possible that the shoots and leaves of lettuce may have been contaminated by virus moving on the external surface of the plant through capillary action. To exclude this possibility, we an identical experiment where the harvested plant tissues were submerged in 50 ml of 1000 ppm chlorine for 5 min was performed. It was found that TV was completed inactivated when incubated with 1000 ppm of chlorine for 2 min (data not shown). Theoretically, treatment of lettuce with 1000 ppm of chlorine for 5 min should

be sufficient to inactivate any virus that may present on the surface of the shoots and leaves. As shown in Fig. 19, there were no significant differences observed in TV internalization in chlorine treated shoots and leaves on any of the study days compared to the untreated samples ($P>0.05$) during the experimental period. However, there was a significant difference in the detection of TV between untreated roots and chlorine treated roots on day 1 ($P<0.05$). Presumably, this is due to the inactivation of the surface virus by chlorine because roots directly contacted the virus-contaminated feed water. However, there were no differences in TV internalization in the chlorine treated roots on days 2, 3, 7, or 14 compared to the roots receiving no treatment ($P>0.05$). This experiment confirmed that TV was indeed absorbed by roots and disseminated to shoots and leaves of lettuce.

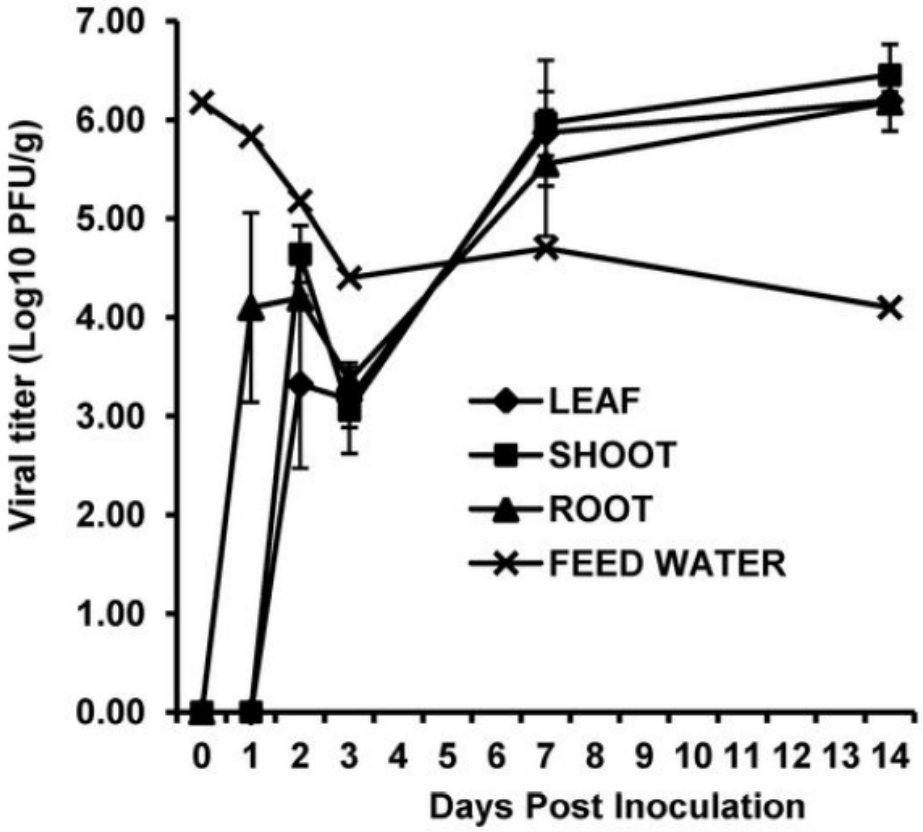

Figure 3. Chlorine treatment of lettuce tissue after TV internalization and dissemination. Viral titer is reported as PFU/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

Internalization and dissemination of MNV-1 in Romaine lettuce grown hydroponically.

The kinetics of MNV-1 internalization in lettuce was also determined. The starting titer (day 0) of MNV-1 feed water in the reservoir for hydroponically grown Romaine lettuce was 2.5×10^6 PFU/ml. The experimental design was identical to that described above. Fig. 4 shows the dynamics of MNV-1 titer in leaves, shoots, roots, and feed water. In leaf tissues, MNV-1 was detected on days 1, 2, 3, 7, and 14 using plaque assays. On day 1, the viral titer detected in

the leaves was 5.9×10^{1} PFU/g and increased to 3.3×10^{5} PFU/g on day 3, and remained at this level for the duration of the study (Fig.4). Similarly, all shoots harvested from days 1 to 14 were positive for infectious MNV-1. On day 1, 5.9×10^{1} PFU/g of MNV-1 was detected in the shoots and increased until day 3 to 3.3×10^{5} PFU/g, and again the level of virus detected in the shoots remained stable until day 14 (Fig. 4). All plaque assay results for roots were positive. MNV-1 was detected in the roots on day 1 at 6.5×10^{3} PFU/g and increased until day 3 to reach a titer of 2.5×10^{5} PFU/g, and the MNV-1 titer was maintained in the roots until day 14 (Fig. 4). MNV-1 titer in the feed water gradually decreased. The initial titer (day 0) in feed water was 2.5×10^{6} PFU/ml. On day 1, the titer decreased to 2.5×10^{5} PFU/ml and on day 2, the titer was further decreased to 2.5×10^{4} PFU/ml, and maintained similar titer until day 14 (Fig. 4). As a control, MNV-1 titer was not significantly decreased in feed water without lettuce (data not shown). This result indicates that the decreasing titer in feed water of growing lettuce was due to the internalization of MNV-1 via roots of lettuce, and not to the instability of MNV-1 in feed water. The starting titer of the feed water of both TV and MNV-1 was comparable. However, there was a significantly higher titer of TV detected in the roots on days 1, 7, and 14 compared to MNV-1 titer in roots. The TV titer detected in the shoots on days 7 and 14 was also significantly higher than the MNV-1 titer detected in shoots. However, the TV titer in the leaves was only significantly higher than MNV-1 on day 7. These results indicate that TV was more efficient in attachment, internalization, and dissemination in lettuce than MNV-1.

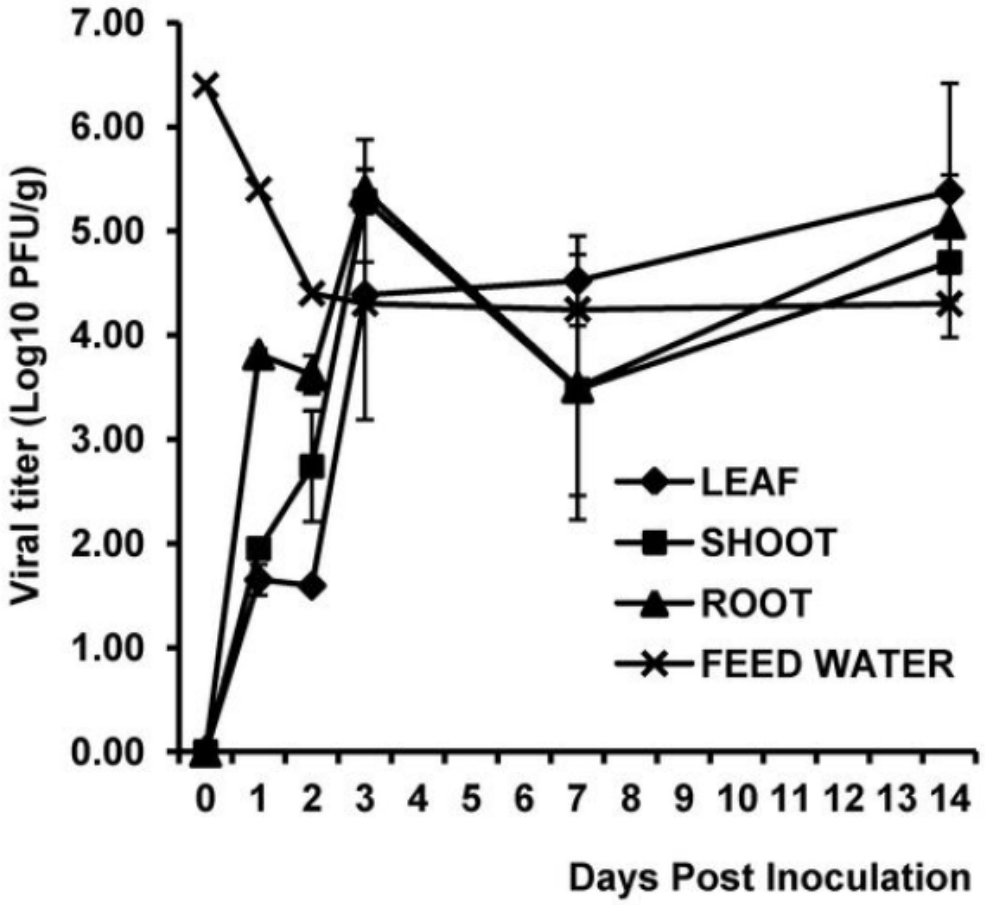

Figure 4. Internalization of MNV-1 in Romaine lettuce grown hydroponically. Internalization kinetics plot was determined by plaque assay and results are reported as PFU/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

Internalization and dissemination of human NoV in Romaine lettuce grown hydroponically

To determine the rate of human NoV internalization, Romaine lettuce was grown hydroponically and the feed water source was inoculated with human NoV GII.4 isolate 5M at a starting titer of 2.9×10^6 RNA. The experimental design and procedures were identical as

described above. The kinetics of viral RNA in leaf, shoot, root, and feed water was quantified by real-time RT-PCR. A high level of human NoV RNA (6.9×10^5 RNA copies/g) was detected in the leaf tissue of the lettuce on day 1 post inoculation and the human NoV RNA detected in the leaves remained stable over the 14 day study period (Fig. 5). Human NoV RNA was also detected in the shoots of lettuce on day 1 post inoculation at a titer of 2.1×10^6 RNA copies/g (Fig. 5), which was significantly higher than that in leaves ($P<0.05$). Similarly, the RNA copies detected in the shoots remained stable over the study period to a final titer of 4.4×10^5 RNA copies/g on day 14 (Fig. 5). Root samples were also positive for human NoV RNA on day 1 post inoculation with a titer of 3.9×10^5 RNA copies/g (Fig. 5). The human NoV RNA detected in the roots reached a peak titer (3.15×10^6 RNA copies/g) at day 3 post-inoculation and decreased to 1.95×10^4 RNA copies/g on day 14. The human NoV RNA copies present in the feed water gradually decreased to a final titer of 1.8×10^5 RNA copies/mL on day 14 (Fig. 5). These results demonstrated that human NoV was efficiently internalized and disseminated in lettuce grown hydroponically.

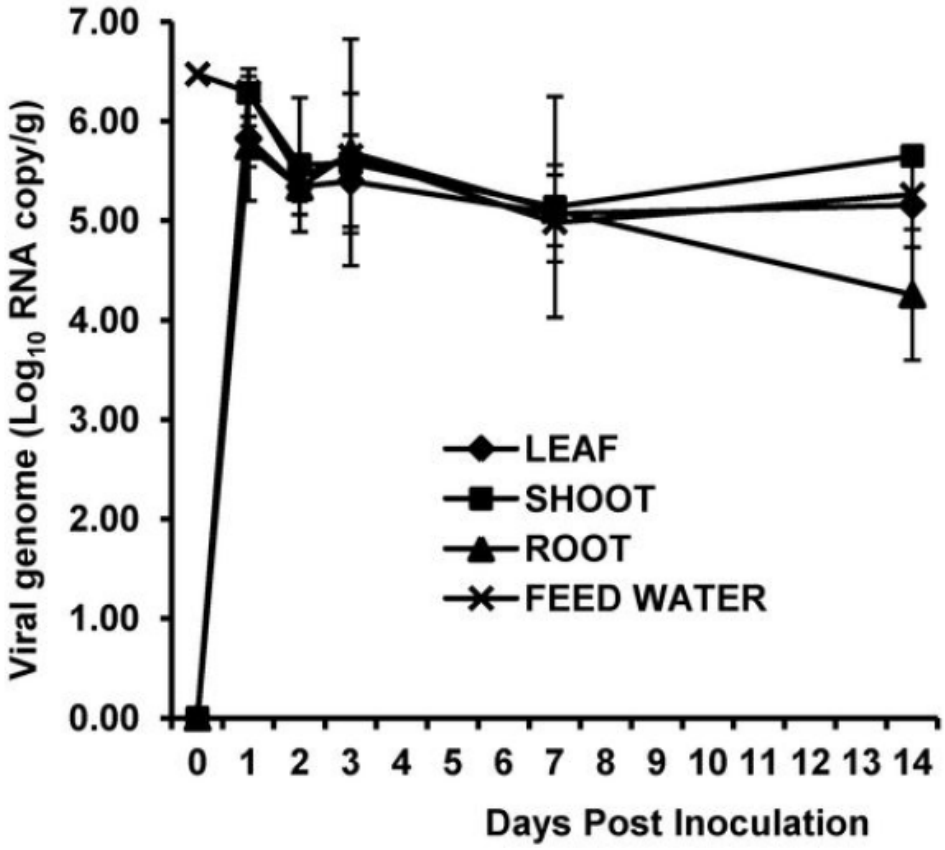

Figure 5. Detection of internalized human NoV GII.4 RNA in Romaine lettuce grown hydroponically. Internalization kinetics plot was determined by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

Subsequently, the internalization rate between human NoV and Tulane virus was compared. Tulane virus was quantified by both plaque assay and real time RT-PCR. As shown in Fig. 6, Tulane virus RNA was detected at a high titer in the leaves (1.9×10^6 RNA copies/g) on day 1 post inoculation and remained stable over the 14 day study period. Similarly, the RNA detected in the shoots was also detected at day 1 post inoculation at a titer of 1.2×10^6 RNA

copies/g (Fig. 6). The TV RNA detected in the shoots also remained stable over the 14 day study period, with no significant change in the RNA detected throughout the study ($P>0.05$). TV RNA was also detected in the roots of lettuce on day 1 post inoculation at a titer of 3.2×10^6 RNA copies/g (Fig. 6). The RNA titer found in the roots remained stable over the 14 day study period, and was similar to the results obtained for RNA copy in the leaf and shoot tissue. Tulane virus RNA copies present in the feed water gradually decreased to a final titer of 1.8×10^5 RNA copies/ml on day 14 (Fig. 6).

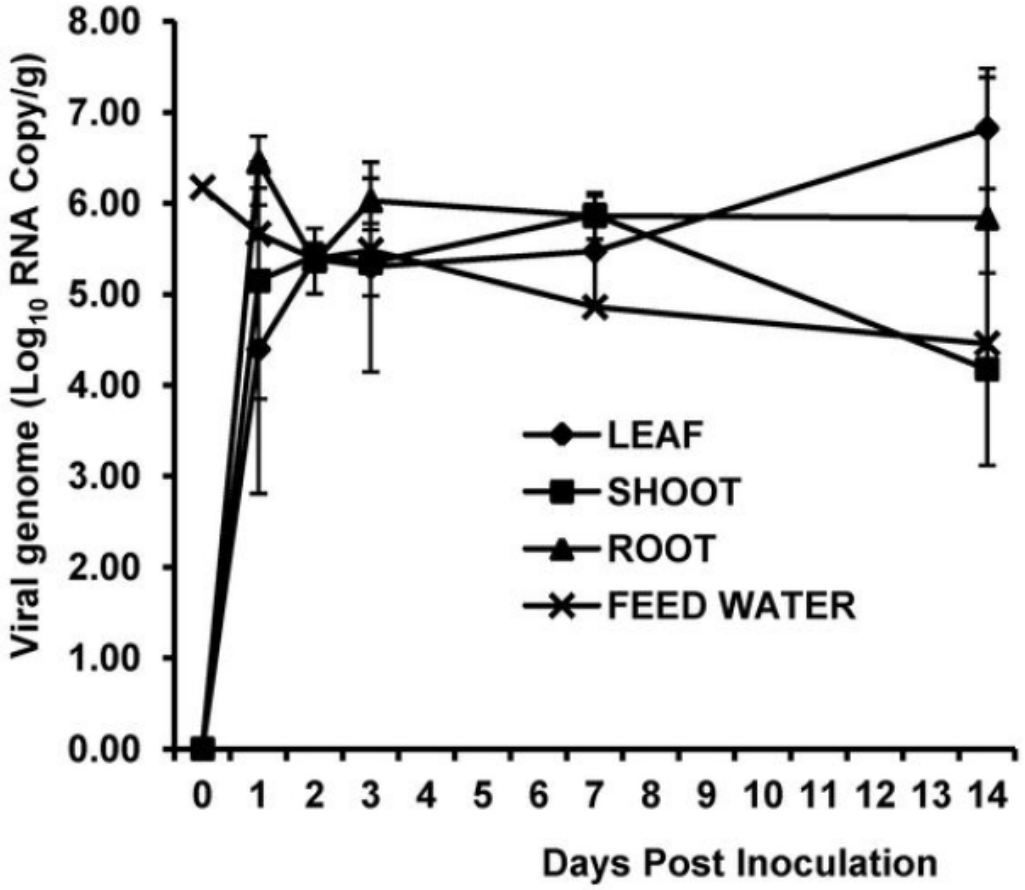

Figure 6. Detection of internalized TV RNA in Romaine lettuce grown hydroponically. Internalization kinetics plot was determined by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation.

Upon comparison it was realized there was a difference in kinetics of Tulane virus internalization determined by the two detection methods, real time RT-PCR and plaque assay. A higher level of Tulane virus RNA (2.5×10^4 to 1.4×10^4 RNA copies/g) was detected in leaves and shoots at days 1 and 2 post inoculation using real-time RT-PCR, compared to a relatively low level of infectious viral particles (1-3 log PFU/g) in leaves and shoots at days 1 and 2 using

plaque assay. It was hypothesized that there may be noninfectious viral particles or naked RNA present in leaves and shoots at days 1 and 2. To address this possibility, all of the samples were treated with 5 µg of RNase A to degrade any exogenous RNA before RNA extraction, and viral RNA was then quantified by real-time RT-PCR. In all day 1 samples tested there was an approximately 2.5 log reduction in the amount of Tulane virus RNA detected in the roots after RNase treatment, compared to samples that were not treated with RNase (Fig. 7). Also, day 1 shoots treated with RNase had approximately 1.3 log reduction in viral compared to untreated samples (Fig. 7). On all other study days tested, there was less than a 1 log reduction in Tulane virus RNA detected due to RNase treatment (Fig. 7). This indicates that some naked viral RNA was present in the plant tissues which was degraded by RNase treatment. It is likely that the naked viral RNA originated from the virus particles which were damaged within the plant tissues.

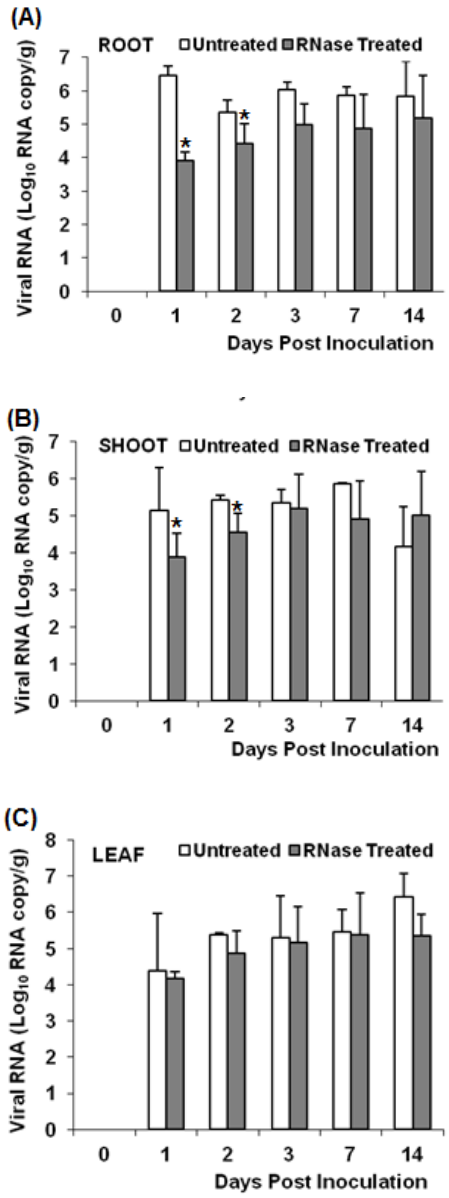

Figure 7: Detection of internalized TV RNA in Romaine lettuce treated by RNase. Viral RNA was quantified by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation and * denotes statistical difference.

To determine whether the amount of human NoV RNA detectable in lettuce samples was affected by pre-treatment with RNase, the same RNase treatment used on Tulane virus lettuce samples was applied to human NoV samples. In contrast to TV, pre-treatment with RNase did not have a significant effect on the amount of human NoV RNA that was detected in the day 1 root samples (Fig. 8). There was approximate 1 log reduction in the viral RNA detected in the shoots after treatment with RNase (Fig. 8). In both TV and human NoV samples, there was not a significant reduction of the amount of RNA detected in the leaf tissue on day 1 post inoculation (Fig. 6, Fig. 8). The RNase treatment reduced the amount of viral RNA detected in the plant tissues by approximately 0.5-1.5 log on all other study days (Fig. 8). Finally, we increased the RNase treatment level to 25 μg, with the same sample concentration and incubation period as above with treatment the of 5 μg of RNase (data not shown).

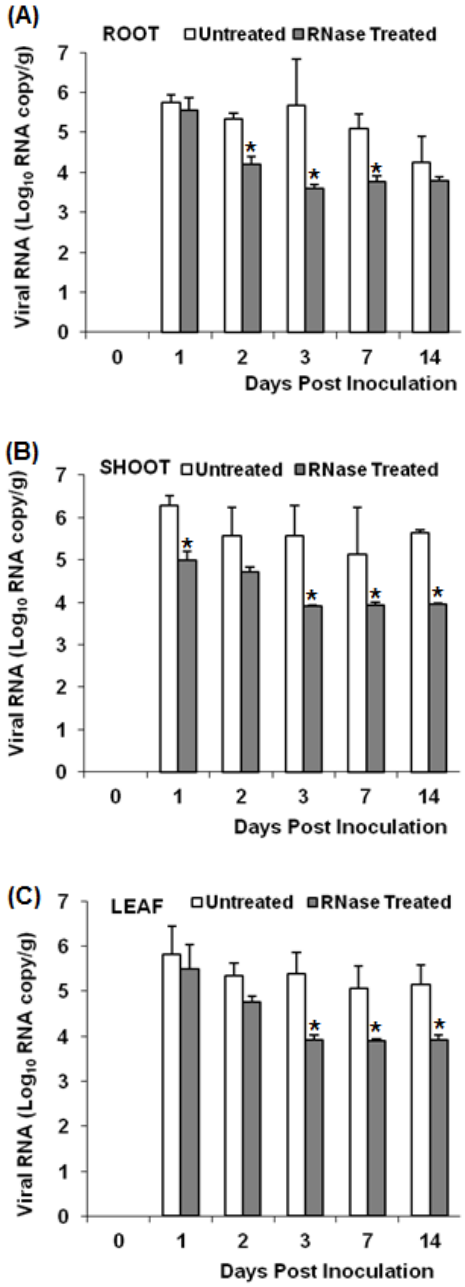

Figure 8. Dectection of internalized human NoV RNA in Romaine lettuce treated by RNase. Viral RNA was quantified by RT-qPCR and results are reported as RNA copies/g. Data points were the averages of three replicates. Error bars represent +/- 1 standard deviation and * denotes statistical difference.

These results indicate (i) the levels of human NoV RNA detected are from a mixture of intact viral particles and naked viral RNA in the plant tissues; (ii) RNase treatment degraded the naked viral RNA; and (iii) intact virus particles persisted in plant tissues for at least 14 days.

Discussion

Human NoV is the leading causative agent of fresh produce-associated outbreaks. However, the interaction of human NoV with fresh produce is poorly understood. In this study, we experimentally demonstrated that human NoV and its surrogates attached to roots, became internalized, and efficiently disseminated to the shoots and leaves of the plants using hydroponically grown Romaine lettuce as a model. Although it has been documented that a low level of internalization and dissemination of MNV-1 and CaCV occurrs in lettuce (Urbanucci et al., 2009, Wei et al., 2010), this is the first report of the successful detection of internalization and dissemination of human NoV in plants.

Fresh produce is one of the major high risk foods for human NoV contamination because it can become contaminated at any point during processing, including both pre-harvest and post-harvest stages. These results indicate that viral internalization through the roots may be an important route for human NoV contamination and persistence in fresh produce. Previously, it was shown that hepatitis A virus RNA could be detected inside green onions which were grown hydroponically in feed water inoculated with this virus (Chancellor et al., 2006). Poliovirus was found in leaves of tomato plants after growth in soil irrigated with poliovirus contaminated water at level of 10^3-10^4PFU/ml. (Oron et al., 1995). Bacteriophage f2 was also detected in beans grown hydroponically when challenged with 10^{10}PFU/ml of the virus (Ward and Mahler, 1982). These results indicate that viral internalization during hydroponic growth of crops does occur,

although the level of virus detected varies among experiments. Since human NoV may be present in sewage-contaminated soil or water, it may also be taken into the plant through the roots. Once viruses are internalized, it would be significantly more challenging to eliminate them, since traditional sanitation measures usually target the pathogens on the surface of fresh produce. Of further concern is that these internalized viruses can potentially survive for long periods (weeks to months) in fresh produce since human NoV is highly stable in the environment.

During either the pre-harvest, or field growing stage of produce production, the use of irrigation water contaminated with norovirus poses the most significant risk in disseminating disease. Agriculture is responsible for the largest usage of freshwater worldwide and about 70% of this usage is for irrigation. Nearly 17% of all cropland is irrigated, which equates to one third of the world wide food supply being exposed to irrigation water (Bosch, 1983). The use of feces or fecally contaminated irrigation water has been shown to play a role in spreading enteric microorganisms. For this reason, the use of night soil or irrigation with untreated human waste water is illegal in the U.S. and is not recommended by the World Health Organization. However, nearly 70% of all the irrigated crop land is found in developing countries where irrigation water regulations may not exist (Choi et al., 2004). Groundwater is generally regarded as being free of microbial contamination and is considered a safe source of irrigation water. However, recent studies in the U.S. indicate that 8-31% of ground water is contaminated with viruses (Abbaszadegan et al., 2007, Borchardt et al., 2003). While irrigation water is commonly screened for fecal coliforms, it is rarely tested for the presence of viruses. All these factors contribute to irrigation water posing a significant risk for distributing viral pathogens to fresh produce.

Previously, Urbanucci et al., (2009) investigated the internalization of human NoV in lettuce. However, no viral RNA was detected in leaves when lettuce was grown hydroponically or in soil after challenge with a high level (10^{6-7} RNA copies/ml) of human NoV. In contrast, in this study it was found that high level of human NoV RNA was detected at day 1 and was persistent in roots, shoots, and leaves at least for 14 days when the roots were challenged with a 10^6 RNA copies/ml of human NoV. Several factors may be responsible for this apparent discrepancy. One possibility is that variations in the experimental conditions between studies, such as, environmental growth conditions, the type of lettuce tested, viral strain, and the amount of viral inoculum. In this study, Romaine lettuce was used whereas Rapid lettuce was used in Urbanucci's study (2009). It is known that environmental factors (such as temperature and relative humidity conditions) have an affect on the transpiration rate of the lettuce, which may have a significant effect on viral internalization and dissemination. In our experiments, the plants were grown at 20^{o}C at relative humidity of 40% but, the growth conditions were not reported in Urbanucci's study (2009). Thus, we cannot directly compare if these environmental factors contributed to the difference in results. Plant transpiration rate increases as the relative humidity of the air decreases, and this increase in transpiration seems to correlate to an increase in viral internalization and dissemination. For example, Wei et al., (2011) showed a significant increase in MNV-1 internalization in lettuce when the relative humidity was 80% compared to 95%. In our study, we decreased the relative humidity to 40%, and the dissemination of MNV-1 to leaves was increased to 4-5 log PFU/g (Fig. 4) compared to the results reported by Wei et al, (2009) at 80% relative humidity.

Similar to bacterial internalization, it is also possible that different viral strains may have differing rates of internalization and dissemination. In our study, we used a genogroup II

genotype 4 (GII.4) strain of human NoV. Although Urbanucci et al. (2009) also used a GII virus, the specific genotype was not reported in their study (Urbanucci et al., 2009). Within genogroup II, at least 33 human NoV genotypes have been identified (Zheng et al., 2006). It is well known that different human NoV genotypes have different binding affinity to its functional receptor, the histo-blood group antigens (HBGAs) (Huang et al., 2005, Hutson et al., 2002, Tan and Jiang, 2001). HBGAs are carbohydrate complexes that are present on the surface of erythrocytes as well as the intestinal, genitourinary, and respiratory epithelia. There are three major families of HBGAs, Lewis, ABO, and secretor, and each is specifically recognized by different human NoV strains. Recent studies have shown that human NoV binds to HBGA-like molecules which exist in fresh produce (such as lettuce, blueberries, and strawberries) (Gandhi et al., 2010, Tian et al., 2007). In fact, with some carbohydrate moieties, the analogues of human NoV receptors, such as glucose and glycan, are highly abundant in vegetables and fruits. It is possible that these HBGA-like molecules may play a role in viral attachment, internalization, and dissemination.

A recent study by Esseili et al. (2012), demonstrated that human NoV GII.4 virus-like particles (VLPs) bound to the cell wall material of young and old leaves, the green leaf lamina, and also the principle vein of Romaine lettuce. This binding was found to be strongest in the cell wall material of old leaves and the green leaf lamina, compared to other plant tissues tested. This was believed to be due to the fact that the cell wall of older leaves are more complex and contain a higher carbohydrate concentration compared to younger leaves. To further demonstrate that the human NoV VLPs were binding to carbohydrates, sodium periodate treatment was used to oxidize carbohydrates in the cell wall extract and this treatment significantly reduced the binding efficiency of the human NoV VLPs (Esseili et al., 2012). The fact that human NoV GII.4 VLPs have been shown to attach specifically to carbohydrates found

in Romaine lettuce may explain the high amount of bioaccumulation of human NoV GII.4 RNA observed in this study. This possibility is further supported by the fact that that HBGA-like receptors exist in gastrointestinal epithelial cells of oysters, mussels, and clams which are also a high risk food for human NoV contamination (Maalouf et al., 2011, Tian et al., 2007). These HBGA-like receptors were shown to play an essential role in bioaccumulation of human NoV in oysters, mussels, and clams (Le Guyader et al., 2006, Maalouf et al., 2011,Tian et al., 2007). Furthermore, different human NoV strains are known to have different binding affinities to shellfish because of their differences in receptor usage. In this study, we also demonstrated that human NoV and TV have similar efficiency in internalization and dissemination in lettuce (Fig. 5, Fig. 6) under the same experimental conditions, whereas TV appears to have a much higher internalization rate than MNV-1 (Fig. 2, Fig. 4). The difference in internalization kinetics may also be related to the properties of each virus such as surface structure, receptor binding affinity, and charge. A recent study has shown that TV also binds to HBGAs, the functional receptor of human NoV (Farkas et al., 2010), but further studies are required to identify whether receptor binding contributes to the bioaccumulation of human NoV in fresh produce.

Since human NoV is not cultivable in cell culture, real-time RT-PCR is frequently used for the detection of human NoV. The major disadvantage of real-time RT-PCR is that it cannot discriminate infectious and noninfectious particles. Thus, one may argue that the high level of RNA copies detected in lettuce may due to the presence of naked human NoV RNA, rather than infectious viral particles. To rule out the possibility, we treated all samples with RNase to degraded naked viral RNA, followed by RNA extraction and real-time RT-PCR. RNase treatment decreased 0.5-1.5 log of human NoV RNA copies in lettuce tissues from days 2 to 14, suggesting that naked human NoV RNA is present in these samples which may come from

damaged human NoV particles (Fig. 8). High levels of Tulane virus RNA were detected in leaves and shoots at days 1 and 2 post inoculation (Fig. 7), whereas low levels of infectious viral particles were isolated from leaves and shoots at days 1 and 2 using plaque assay (Fig. 2). After RNase treatment, there was an approximate 2.5 log reduction in the TV RNA detected in the shoots (Fig. 7). In leaves harvested on day 1, there was $2.5x10^4$RNA copies/g detected by real time RT-PCR (Fig. 7), whereas less than 1 log of infectious virus was detected by plaque assay (Fig. 2). However, RNase treatment did not significantly reduce the amount of TV RNA detected in leaves ($P>0.05$) indicating that some noninfectious viral particles, and not naked RNA, were present in the leaves. A similar phenomenon was observed by Wei at al. (2011), where MNV-1 RNA, but not infectious MNV-1, was detected in the leaves of Romaine lettuce inoculated with MNV-1 at the root juncture. It is likely that these viral particles were damaged and hence rendered noninfectious, whereas the viral RNA persisted in the plant tissues. Although plants lack an immune system analogous to the human immune system, plant have developed an array of structural, chemical, enzymatic, and protein-based defenses aimed at detecting and eliminating invading organisms (DeWit 2007, Postel and Kemmerling, 2009). It is possible that different stability against these varying plant defenses. Therefore, the most compelling data presented in this study may be on the rate of human NoV internalization and dissemination in Romaine lettuce, compared to the data from human NoV surrogates.

In summary, our study elucidates a major gap in our understanding of ecology of human NoV in fresh produce, specifically, our understanding of the fate of human NoV after attaching to roots of growing lettuce. Dissection of the mechanism of virus-plant interactions will facilitate the development of novel interventions to prevent viral attachment and internalization in plants.

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